Characterization of Opioid Receptor Types and Subtypes with New Ligands

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Opioid drugs and opioid peptides produce their behavioral effects, including antinociception, by interactions with opioid receptors in the central nervous system. The existence of specific opioid receptors was originally suggested by behavioral and clinical studies, and was confirmed by biochemical identification (*in vitro* binding experiments) in 1973.[1-4] Since then, extensive studies have been undertaken on their localization, biochemical, and pharmacological characterization.

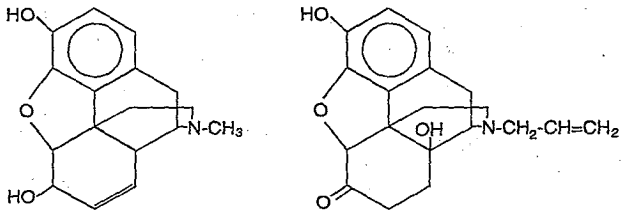
The classical ligand for opioid receptors is morphine (FIG. 1.), originating from the alkaloids of the poppy plant. As far as the structural requirements are concerned for opiate action, the presence of a phenolic OH group in position 3 is important. The substitutions on the nitrogen determine the agonistic or antagonistic character of the ligand: the methyl group results in agonistic properties, whereas the allyl group (as in the case of naloxone) leads to an antagonistic character. The allyl group can be replaced by a cyclopropylmethyl or propyl group (e.g., naltrexone, or N-propylnoroxymorphone).

The first endogenous ligands for opioid receptors were identified in 1975 by Hughes *et al.*[5] The N-terminal Tyr of the two pentapeptides (methionine- and leucine-enkephalins) correspond to the A ring of morphine (FIG. 2). A number of other endogenous opioid peptides have been described in the meantime. A representative list of them is outlined in TABLE 1. Most of these compounds show a homology—the first four residues are identical. This structural arrangement can easily be explained by the "message-address" concept, originally described by Schwyzer.[6] The N-terminal region of the molecule is constant and carries the message, whereas the other part is fairly variable, resulting in functional heterogeneity. Accordingly, opioid receptors are heterogeneous, consisting of at least three major types, mu, delta, and kappa. The major opioid receptor types, their representative ligands, and effects are shown in TABLE 2. The three opioid receptors exhibit different ligand selectivity profiles. Most endogenous opioids and synthetic ligands do not possess absolute specificity for a given receptor type, but can interact with more than one opioid receptor type. The situation is further complicated by the fact that multiple receptor types may coexist within a single tissue, or even in a cell. Although the multiplicity of opioid receptors is generally accepted, the molecular basis of the heterogeneity is not completely understood. The primary structure of the delta receptor cDNA was reported simultaneously by Kieffer *et al.*[7] and Evans *et al.*[8] at the end of 1992, followed by the cloning of the mu[9] and kappa receptor.[10] Further multiplicity has not been proved yet by the cloning experiments.[11,12]

A better understanding of multiple opioid receptor structures and functions is of great importance for both the theoretical and practical points of view. Over the last few years increasing attention has been focused on studies of the heterogeneity of the opioid receptor types and especially on opioids acting at the delta as well as the

OPIOID

AGONIST ANTAGONIST

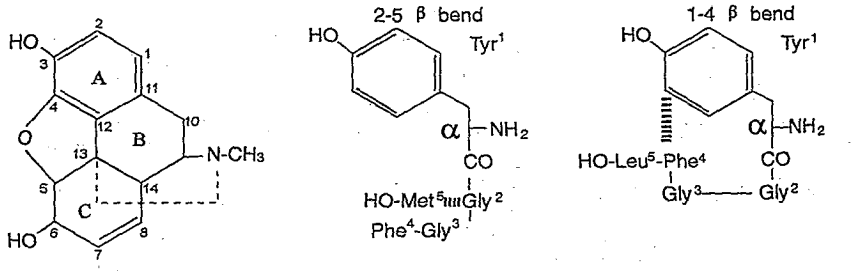


MORPHINE NALOXONE

FIGURE 1. Chemical structure of classical opioid ligands.

kappa receptors, because these compounds may cause fewer side effects than mu opioid receptor agonists do, thereby possibly providing an attractive alternative to the currently used opioid analgesics. Several attempts are presently under way in various laboratories to develop highly specific compounds, the use of which is crucial for understanding the mechanism of opioid action at the level of the endogenous system, in neurochemical processes in various mental diseases and pain states, and will be of direct benefit in improved therapy.

Opioid drugs are, and will continue to be, essential therapeutic agents. They provide the ultimate treatment for pain, but their use is complicated by many other effects. The most notable ones include respiratory depression, sedation, and gastrointestinal dysfunction. Chronic use of opioids can also result in addiction and physical dependence. The production of compounds selective for the opioid receptor types/subtypes may provide the means to safe analgesics. The observation that distinct receptor types may mediate different nonanalgesic effects opened the possibility that some opioid side effects might be avoided by more selective drugs acting on different opioid receptor populations. The important role of mu opioid receptors in the development of opioid tolerance and physical dependence is well



MORPHINE MET-ENKEPHALIN LEU-ENKEPHALIN

FIGURE 2. Comparison of structure of ± morphine and enkephalins.

TABLE 1. Endogenous Opioid Peptides

Precursor	Opioid-Peptide	Structure	Selectivity
Pro-opio-melanocortin (POMC)	β-Endorphin	*Tyr-Gly-Gly-Phe-Met*-Thr-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys- Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu	μ, ε > δ ≫ κ
Pro-enkephalin A	[Leu^5]Enkephalin	*Tyr-Gly-Gly-Phe-Leu*	δ > μ ≫ κ
	[Met^5]Enkephalin	*Tyr-Gly-Gly-Phe-Met*	μ ~ δ ≫ κ
	[Met^5]Enkephalin-Arg^6-Phe^7	*Tyr-Gly-Gly-Phe-Met*-Arg-Phe	κ_2
Pro-dynorphin (Pro-enkephalin B)	Dynorphin A (1–17)	*Tyr-Gly-Gly-Phe-Leu*-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gly	κ ≫ μ > δ
	Dynorphin A (1–13)	*Tyr-Gly-Gly-Phe-Leu*-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys	κ > δ ~ μ
	Dynorphin A (1–8)	*Tyr-Gly-Gly-Phe-Leu*-Arg-Arg-Ile	κ > δ ~ μ
Others			
β-Casein derivatives	Morphiceptin	Tyr-Pro-Phe-Pro-NH_2	μ
	β-Casomorphin	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	
α-Gliadin derivatives	Gliadorphin	Tyr-Pro-Gln-Pro-Gln-Pro-Phe	
Frog brain peptides			
Phyllomedusa sauvagei	Dermorphin	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	μ
	Deltorphin	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	δ
Phyllomedusa bicolor	[D-Ala^2]Deltorphin I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	δ
	[D-Ala^2]Deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	δ

documented. However, the involvement of delta opioid receptors in the development of these adaptive phenomena is less known.

Opioid antagonists have been indispensable pharmacological tools for identifying receptor types involved in the interaction with endogenous and synthetic opioid agonists. The antagonists are especially useful in the case, when the pharmacological endpoints are identical (e.g., antinociception or inhibition of a smooth muscle preparation by agonists), and it is not easy to distinguish among mu, delta, and kappa opioid receptor mediated agonist effects.

In this review we describe the characteristics of a number of new opioid ligands prepared in normal and in tritiated forms.

TABLE 2. Heterogeneity of Opioid Receptors

	μ	δ	κ
	β-end > dynA > met > leu[a]	met = leu > β-end > dynA[a]	dynA ≫ β-end > leu = met[a]
Selective agonists	DAMGO Sufentanyl PLO17	DPDPE DSBULET [D-Ala²]Deltorphins	U69593 CI977 ICI197067
Selective antagonists	CTAP Cyprodime	ICI174864 Naltrindole TIPP	Nor-binaltorphimine
Radioligands	[³H]DAMGO [³H]PLO17	[³H]DPDPE [³H]TIPP [³H]Naltrindole	[³H]U69596 [³H]CI977
Predominant effectors	cAMP ↓ K^+ channel ↑ Ca^{2+} channel ↓	cAMP ↓ K^+ channel ↑ Ca^{2+} channel ↓	cAMP ↓ K^+ channel ↑ Ca^{2+} channel ↓
Structural information	398 aa rat, mouse 7TM	372 aa rat, mouse 7TM	380 aa rat, mouse 7TM

[a]Potency order.

MU RECEPTOR-SPECIFIC LIGANDS

Agonists

The clinically employed opioid alkaloids (morphine, methadone, fentanyl, etc.) preferentially bind to the mu receptors and have a high potential for abuse. Their use is limited because of the development of tolerance and dependence and other side effects (respiratory depression, etc.). Recently a new group of compounds (14-alkoxymetopon derivatives) was described[13] with reduced dependence liability. It was shown earlier that the introduction of a 14-metoxy group to N-methylmorphinane-6-ones leads to a dramatic increase in antinociceptive potency.[14] A number of 14-alkoxymetopons (FIG. 3) have been tested in biochemical and pharmacological assays.[13] It was shown that the new ligands exhibited high affinity towards the mu sites. The sodium indexes were found to be extremely high (between 41–133), reflecting the agonist property of the compounds. This was further proved on isolated guinea-pig longitudinal muscle preparation, where the relative potency was 48–72 times higher than that of normorphine. The naloxone reversible antinocicep-

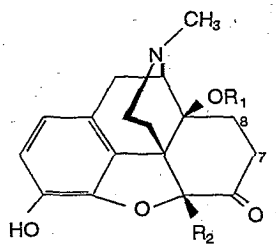


FIGURE 3. Chemical structure of alkoxymetopons.

(1) $R_1 = R_2$=H (OXYMORPHONE);
(2) $R_1 = R_2$=CH_3 (14-METHOXYMETOPON);
(3) R_1= C_2H_5, R_2=CH_3 (14-METHOXYMETOPON);
(4) $R_1 = R_2$=CH_3, 7.8 (14-METHOXY-5-METHYLMORPHINON).

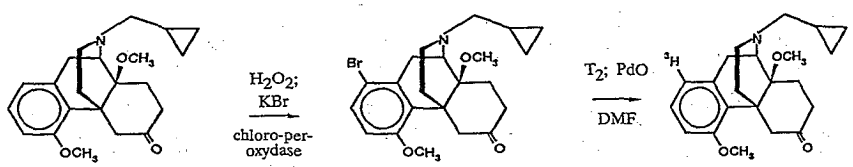
tive effects in rats and mice were 130–300 times higher than in the case of morphine. Moreover, the dependence liability of the 14-alkoxymetopon derivatives in the withdrawal jumping tests was less pronounced than that of morphine (38–78% of control) in both species. It was also found by our laboratory that a major side effect—respiratory depression—of opioid alkaloids is diminished using certain codeinone analogues, which still hold analgesic potency[15] (see more detailed description under the section AFFINITY LABELING).

Antagonists

Cyprodime is known to be the only pure mu antagonist ligand among the heterocyclic compounds.[16] It has recently been radiolabeled[17] according to the schematic representation shown in FIGURE 4. This enzymatic procedure was chosen to have a brominated precursor that was followed by catalytic dehalogenation methods to obtain theoretical specific radioactivity. The complete biochemical characterization of this radioligand is currently in progress.

DELTA RECEPTOR-SPECIFIC LIGANDS

Pharmacological evidence has suggested the existence of two delta receptor subtypes in the brain in 1991.[18,19] From the known delta-specific ligands, [D-Pen^2,



cyprodime — 1-*bromocyprodime* — [1-^{3}H]cyprodime

31.6 Ci/mmol

FIGURE 4. Tritiation of the mu-specific opioid antagonist, cyprodime.

TABLE 3. Selected Endogenous Opioid Peptides from Frog Skin

Dermorphin TYR-D-ALA-PHE-GLY-TYR-PRO-SER-NH_2
Deltorphin TYR-D-MET-PHE-HIS-LEU-MET-ASP-NH_2
Deltorphin I TYR-D-ALA-PHE-ASP-VAL-VAL-GLY-NH_2
Deltorphin II TYR-D-ALA-PHE-GLU-VAL-VAL-GLY-NH_2

D-Pen^5]enkephalin (DPDPE)[20] is thought to be primarily an agonist at the opioid $delta_1$ subtype, whereas [D-Ala^2, Glu^4]deltorphin is a selective agonist at the $delta_2$ subtype.[21]

Agonists

Linear hexapeptides, DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) and DTLET (Tyr-D-Thr-Gly-Phe-Leu-Thr), have been developed in the laboratory of Roques. Recently, researchers there introduced the lipophylic and bulky tert-butyl group on the Ser^2 and Thr^6 amino acids of DSLET. The resulting new compounds (DSBULET, BUBU, and BUBUC) are highly potent and selective full agonist of delta receptors.[22] BUBU and BUBUC are protected from peptidases and were recently shown to be able to enter the brain,[23] allowing for the first time the effects resulting from delta receptor stimulation to be investigated after systematic administration, that is, in clinically relevant conditions. BUBU and BUBUC display interesting antidepressant-like properties and spinal analgesic activity without cross tolerance to morphine in chronic pain.

Following the isolation of a heptapeptide (dermorphin) from frog (*Phyllomedusa sauvagei*) skin,[24] another peptide (deltorphin) was found by recombinant DNA technology.[25] Both peptides derive from a common, larger precursor and contain amino acids with a D configuration in position 2. Later two more peptides were identified in another frog species[26] and were found to be excellent ligands for the delta receptor type (TABLE 3).

Buzas *et al.* prepared and characterized the $delta_2$-specific peptide deltorphin II in tritiated form.[27] Lately, new analogues of this compound have been synthesized, with the aim of better specificity and affinity towards the $delta_2$ sites. Among them, Ile residues were incorporated into the fifth and sixth position of deltorphin II[28] and radiolabeled using a diiodo-Tyr containing precursor peptide[29] (TABLE 4). The presence of the more hydrophobic residues resulted in an increased affinity (K_d: 0.4

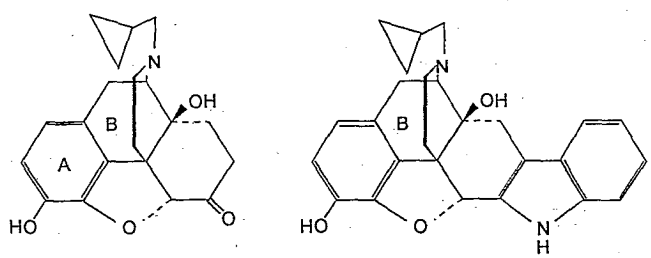
TABLE 4. Tritiation of Deltorphin Analogues

Peptide	Catalyst	Specific Activity	
		GBq/mmol	(Ci/mmol)
[p-^{3}H-Phe^3]Deltorphin II	PdO	726	(20.6)
[p-^{3}H-Phe^3]Deltorphin II	PdO/$BaSO_4$	908	(24.5)
[3′,5′-^{3}H-Tyr^1,Ile^{5,6}]Deltorphin II	PdO/$BaSO_4$	2364	(63.9)

nM) and selectivity (selectivity ratios: mu/delta 2400; kappa/delta 18000). Further advantages of this ligand are low, nonspecific binding (<25%) and high, specific radioactivity (TABLE 4).

Antagonists

A potent and moderately selective delta antagonist was developed by Portoghese. The structure of naltrindole (NTI)[30] is based on the morphinane skeleton to which an indole nucleus is fused (FIG. 5). It was shown that the development of acute tolerance and dependence in mice pretreated with NTI before induction of tolerance and dependence with morphine sulfate was markedly suppressed. The use of this delta-opioid receptor antagonist allows a way to prevent opioid tolerance and physical dependence without compromising the antinociceptive activity of mu-opioid receptor agonists such as morphine. Recently, NTI was radiolabeled in one[31] and subsequently in two positions.[32,33] This ligand binds to delta opioid receptors with high affinity, but a significant proportion of the binding becomes wash-resistant. This pseudoirreversible nature of the binding might be due to the hydrophobic property of the ligand.



NALTREXONE NALTRINDOLE

FIGURE 5. Structure of selected opioid antagonists.

Relatively selective antagonists have recently been obtained: [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE)[34] for delta$_1$ and naltrindole-5′-isothiocyanate (5′-NTII)[18] for delta$_2$ receptor subtypes. Several studies suggest that both delta$_1$ and delta$_2$ opioid receptors mediate antinociception in mice.[18,35] Both receptor subtypes appear to mediate antinociception at the supraspinal level, whereas the delta$_2$ receptor is involved in antinociception at the spinal level. Finally, cold-water swim stress produces an opioid mediated antinociceptive response, which appears to be antagonized by 5′-NTII, but not by DALCE. Thus, on the basis of the recent data the existence of different subtypes of delta receptors in the central nervous system of rodents is suggested. This heterogeneity of delta receptors was further supported by ligand binding assays,[19] although not yet supported by the cloning of a single opioid receptor gene coding a protein which binds a delta subtype selective ligand.

Schiller recently designed a peptide antagonist for the delta receptor, based on conformational restriction.[36] The tetrapeptide H-Tyr-Tic-Phe-Phe-OH (TIPP) (FIG. 6) and its tritiated form displays high delta-receptor affinity, unprecedented delta selectivity, high potency as a delta antagonist, and, unlike other delta antagonists,

shows no mu-antagonist properties and is 80 times more selective than NTI.[37] It was shown earlier that antagonists may be obtained by the reduction of the peptide bond (CH_2NH) in the case of mu ligands. This approach has been applied also to obtain delta antagonists. A chemically and enzymatically stable, more potent and more selective analogue, Tyr-TicΨ(CH_2NH)Phe-OH (TIPP[Ψ]) was described very recently[38] and has already been radiolabeled (manuscript submitted).

KAPPA RECEPTOR-SPECIFIC LIGANDS

Kappa opioid receptors play a role in various pharmacological and physiological functions, such as analgesia, behavioral and autonomic effects, regulation of neurotransmitter and hormone release and synthesis, modulation of membrane ion-channels and calcium uptake.[39] The kappa agonists are considered to be advantageous drugs in producing spinal analgesia, for treating rheumatic fever disease, strokes, in reducing chemical, visceral, and thermal stimuli. Moreover, they do not

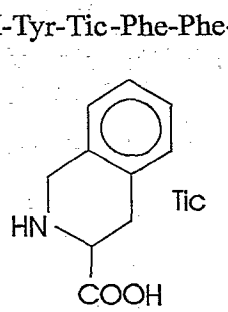


FIGURE 6. Structure of the delta opioid receptor specific peptide, H-Tyr-Tic-Phe-Phe-OH (TIPP). Tic: Tetrahydro isoquinoline-3-carboxylic acid.

induce dependence as mu-specific ligands do, but they may produce dysphoria. In the last few years the heterogeneity among kappa receptors became evident, although their exact role still has to be elucidated. Presently, U-69,593 is considered to be one of the best $kappa_1$ selective ligands. It is important to design, synthesize, and label new ligands for other subtypes as well.

Agonists

Met-enkephalin-Arg^6-Phe^7 was earlier considered as a $kappa_2$ selective ligand.[40] Therefore, [^{3}H]Met-enkephalin-Arg^6-Phe^7 has been synthesized and labeled from a diiodo-Tyr containing precursor peptide with dehalotritiation.[41] The results obtained with this radioligand raised the possibility of further heterogeneity. On the other hand its application is presently limited because of its sensitivity toward various peptidases. For this reason the synthesis of more stable analogues is feasible.

Previous pharmacological experiments showed the analgesic efficacy of the 2,4-dipyridine substituted dimethyl-3,7-diazabicyclo[3,3,1]nonan-9-on-1,5-dicarboxylate[42] (FIG. 7.). It was thought that the effect was the result of an interaction of this

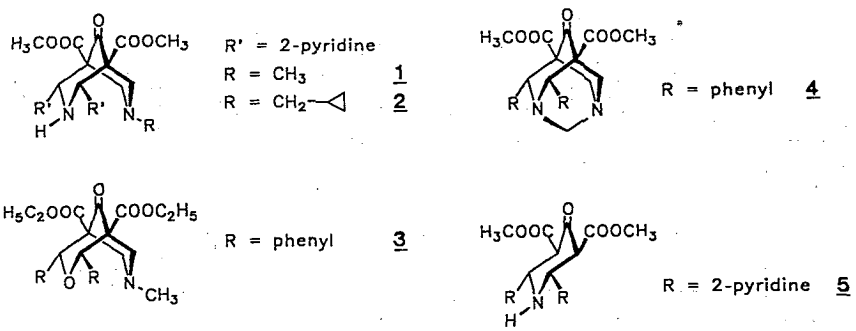
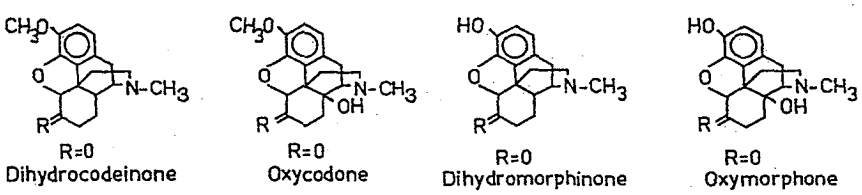


FIGURE 7. Structure of heterocyclic bicyclo[3,3,1]nonan-9-ones.

unusual compound with the kappa receptor type. Very recently it was found that this new class of ligands (heterocyclic bicyclo[3,3,1]nonan-9-ones) exhibit kappa$_1$ agonist selectivity.[43] The further modification of these ligands will allow the study of the structure of opioid kappa receptor subtypes. Besides the theoretical importance, this new group of ligands may lead to the development of potent analgesic drugs with reduced side effects.

AFFINITY LABELING OF OPIOID RECEPTORS

Structural studies of many receptor binding sites were greatly facilitated by the use of affinity ligands, which are capable of covalent interactions with the receptors. A number of these compounds have been developed in normal and tritiated form for the identification of opioid receptors. A large percentage of their binding became



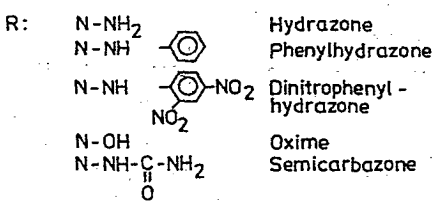


FIGURE 8. Structure of morphinane-6-ones.

irreversible under the proper conditions in rat and frog brain. The new ligands were either modifications of morphine or derivatives of enkephalins.

Morphine Derivatives

Hydrazone, phenylhydrazone, and dinitrophenylhydrazone, oxime, and semicarbazone derivatives of dihydromorphone and oxymorphone (FIG. 8) were prepared.[15] The N-substituted hydrazones and the oxime and semicarbazone derivatives were all capable of irreversible inhibition of [^{3}H]naloxone binding. The blockade concerned mainly the high-affinity binding site. We concluded that the C = N double bond was responsible for the irreversible binding. A tritiated form of oxymorphazone has also been prepared and characterized in binding assays.[44] Among the corresponding codeinone derivatives, selective blockers were found for the low-affinity site. This observation has a pharmaceutical significance, because this site is thought to be responsible for the respiratory depression, which is the major side effect of the morphine derivatives.[45] It was suspected that different mu receptor subtypes may mediate antinociceptive, gastrointestinal, and respiratory actions induced by C-6 substituted oxicodone derivatives. It was found that low doses of the oxycodone derivatives failed to inhibit gastrointestinal transit and resulted in a slight increase of respiratory function. Moreover, the respiratory depression induced by mu agonists (e.g. morphine, fentanyl) was prevented in conscious rabbit and narcotized rat (TABLE 5).

TABLE 5. Effect of C-6 Substituted Oxycodone Derivatives on Arterial Blood Gases in Anesthetized Rats

Treatment	Arterial Blood Gas Measurements	
	pO_2	pCO_2
Fentanyl (10 μg/kg)		
Baseline values	111.1	28.9
Change (%)	−35.5	+69.5
Oxycodone oxime (10 μg/kg)		
Baseline values	107.5	27.1
Change (%)	+42.0	−44.3
Oxycodone oxime + fentanyl		
Baseline values	108.5	31.3
Change (%)	+5.2	−15.5

Enkephalin Derivatives

A number of enkephalin analogues were elongated with a chloromethyl ketone group at the C-terminus, which led to a shift in their specificity from the delta towards the mu site. They were all able to irreversibly block the high-affinity naloxone binding site. A tritiated form of D-Ala^2-Leu^5 enkephalin chloromethyl ketone (DALECK) was used first for the identification of opioid binding sites in rat and frog brain.[46,47] Later, a highly mu-selective compound was prepared from Tyr-D-Ala^2-Gly-(Me)Phe-Gly-ol (DAMGO). The new chloromethyl-ketone deriva-

tive and its radiolabeled form were synthesized by a fragment condensation method (spec. radioactivity 56 Ci/mmol).[48] More recently, hydrodynamic parameters of mu opioid receptors were measured on [^{3}H]DAMCK-prelabeled preparations of rat brain under nondenaturing conditions. The apparent M_r on SDS-PAGE followed by fluorography was found to be 58 kDa,[49] confirming previous data reported in the literature.[50] This size of the mu receptor was shown in various species including rat, guinea pig, rabbit, and chicken (unpublished results). The molecular mass of the cloned receptors is much less because of the lack of the polysaccharide chains. (All of the major opioid receptor types are glycoproteins.)

FUTURE DIRECTIONS

The recently described cloning of the major opioid receptor types will facilitate the development of new compounds (agonists and antagonists) for use in further detailed structural analysis of the receptors and for new clinically useful opioids. The use of antagonists will be inevitable in the transfected cells (containing different second messenger systems); besides, it is likely that there are distinct binding sites for agonists and antagonists.

The novel ligands will be tested by chemical, biochemical, and pharmacological assays for a better understanding of the interactions between the ligands and receptor types/subtypes. The determination of the subtype selectivity of the new compounds is expected, for example, in the case of delta receptors. As stated earlier in this review, there is evidence for the existence of two receptor subtypes, delta$_1$ and delta$_2$. Neither NTI nor TIPP seems to discriminate between these two receptor subtypes. Although structural modification of naltrindole has resulted in antagonists with some preference for both delta$_1$ and delta$_2$ receptors, pure antagonist compounds with further improved selectivity for either delta$_1$ and delta$_2$ receptors have to be developed. Such compounds are absolutely necessary for the definition of the distinct functional roles of these two receptor subtypes. In particular, the availability of such receptor subtype-specific antagonists would also allow us to examine the important question of whether the effect on morphine tolerance and dependence observed with naltrindole is mediated by the delta$_1$ or the delta$_2$ receptor or by both. On the basis of results obtained from *in vitro* binding assays, isolated tissue preparations and pharmacological tests, more candidates will be selected for radiolabeling.

Different chemical approaches can be applied to avoid enzymatic degradation of the peptides and to achieve improved bioavailability. Some of the peptides are modified for obtaining pseudopeptides with restricted conformations and enhanced resistance to peptidases. Based on NMR and theoretical conformational studies, we expected to obtain new information for defining the pharmacophore, corresponding to the receptor types (subtypes). Some of the modifications will lead to radiolabeled compounds, allowing the use of autoradiographic and electronmicroscopic techniques also for studying the receptors. For obtaining ligands with better selectivity and antagonist property, a further reduction in conformational freedom is required. It is expected that conformational restriction of peptides in some cases might also reduce or even totally abolish their intrinsic activity ("efficacy") and, thus, may produce partial agonists or antagonists.

Besides the functional assays, the application of molecular biological techniques (including site-directed mutagenesis and hybridization experiments) will be required for the complete understanding of the effects of the novel ligands in the opioid system. Studies of the regulation of opioid gene expression by the specially synthesized analogues will certainly be performed. It is also expected that regulation of

opioid receptor mRNAs will be estimated by *in situ* hybridization histochemistry. All these approaches are necessary in order to fulfill the ultimate goal of developing new therapeutic agents with fewer undesired side effects.

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